{Exhibit 63}

Voller et al., "A microplate method of enzyme-linked immunosorbent assay and its application to malaria," Bull, W.H.O., 51: 209-211 (1974)

A microplate method of enzyme-linked immunosorbent assay and its application to malaria*

A. VOLLER,[1] D. BIDWELL,[2] G. HULDT,[3] & E. ENGVALL[4]

Abstract

A microplate method of enzyme-linked immunosorbent assay is described. This method, which may be of value in the measurement of antibodies in various infectious diseases, is simple, cheap, and easy to carry out on a large scale. For field use, results can be assessed subjectively. The potential of the test is illustrated by a small seroepidemiological study of malaria.

Engvall & Perlmann (1) described an enzyme-linked immunosorbent assay (ELISA) for antibodies and Voller et al. (2) applied this to malaria, with promising results. These tests were carried out in polystyrene tubes, but this presents some problems for large-scale use and is wasteful of valuable reagents.

The present communication deals with the development of a simple plate method for the assay and with an example of its application to a small seroepidemiological study of malaria.

Materials and methods

Plates. Disposable polystyrene plates (Cooke Microtiter[a] M29 AR) with 96 wells were used as the carrying surface for the antigen.

Antigen. The malarial antigen employed in this study was derived from the infected erythrocytes of *Aotus trivirgatus* with heavy *Plasmodium falciparum* parasitaemia. Blood was taken in heparin from heavily infected monkeys and was cultured by the method of Cohen & Butcher (3). The cultured erythrocytes containing mature parasites were washed 3 times in phosphate-buffered saline (pH 7.2) and were centrifuged at 1 000 *g* for 15 min. After the leucocytes had been removed with a pipette, the upper brown layer of infected erythrocytes was washed in phosphate-buffered saline and recentrifuged. The pellet of schizont-infected cells was the antigen source. It was diluted 1 : 10 in phosphate-buffered saline and was subjected to sonication for 20 s. Debris was removed by centrifugation and the supernatant, which was used as the antigen, was stored in 0.1-ml aliquots at −70°C.

Titration of antigen. A positive reference serum from a person with confirmed *P. falciparum* infection and a negative reference serum from a person never exposed to malaria were used to titrate the antigen. A series of antigen dilutions ranging from 1 : 100 to 1 : 1 000 was used to sensitize the plates. As described below, these were then tested with the two reference sera, which were diluted 1 : 1 000. The highest antigen dilution that gave a strong reading (E400 > 1.0) with the positive serum and a low reading (E400 < 0.1) with the negative serum—in this case, 1 : 250—was used in all subsequent tests.

Sensitization of plates. The antigen was diluted 1 : 250 in carbonate buffer (0.05 mol/l, pH 9.6) with 0.02% sodium azide preservative. Then 0.3 ml of this diluted antigen solution was used to sensitize each well in Cooke Microtiter M29 AR plates, after which they were washed 3 times in saline containing 0.05% Tween 20. After the final wash, the wells were emptied and the plates were ready to receive the test sera.

Conjugate. The conjugate was rabbit antihuman immunoglobulin labelled with alkaline phosphatase by the method of Engvall & Perlmann (1). At a dilution of 1 : 800 it gave strong reactions with the positive reference serum and a low level reaction with the negative reference serum. The undiluted conjugate containing 0.02% sodium azide as a preservative was stored at +4°C.

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* Preliminary communication. A more detailed account will be published elsewhere.

[1] Senior Lecturer, London School of Hygiene and Tropical Medicine, London, England, and Nuffield Institute of Comparative Medicine, The Zoological Society of London, Regent's Park, London, England.

[2] Research Assistant, Nuffield Institute of Comparative Medicine, The Zoological Society of London, Regent's Park, London, England.

[3] Chief, Parasitology Section, National Bacteriological Laboratory, Stockholm, Sweden.

[4] Research Assistant, University of Stockholm, Sweden.

[a] Dynatech Laboratories.

The *substrate* was p-nitrophenylphosphate (1 g/litre), made up in 10% diethanolamine buffer (pH 9.8) containing magnesium chloride (0.5 mmol/l) and 0.02% sodium azide.

Test. The assay procedure was based on that of Engvall & Perlmann (1), but with a constant reaction time and the use of plates instead of tubes.

(1) Sera or plasma were diluted 1 : 1 000 in phosphate-buffered saline containing 0.05% Tween 20 and 0.02% sodium azide.

(2) After 0.3 ml of each diluted sample had been added to separate wells in the sensitized plates, the plates were agitated and then incubated for 2 h at room temperature.

(3) The wells were emptied and washed for 15 min in saline containing 0.05% Tween 20.

(4) The wells were emptied and 0.3 ml of the diluted conjugate was added to each well. After agitation the plates were incubated for 3 h at room temperature.

(5) The washing was repeated as in (3).

(6) Then 0.3 ml of the substrate solution was added to each well. After having been agitated, the plates were incubated at room temperature for 30 min.

(7) The reaction was stopped after 30 min by the addition of 0.05 ml of sodium hydroxide (2 mol/l) to each well.

The absorbance at 400 nm (E400) of the contents of each well was determined on a spectrophotometer. A visual assessment of the plates was also made, those with an obviously yellow colour being considered as positive.

Samples tested. The plasma samples tested in the present study were derived from people living in two different areas of Colombia. In one area, San Juan de Arama, there was current transmission of *P. vivax* and *P. falciparum*. In the other, Girardot, an eradication scheme has been in progress for some years. Blood was obtained by finger-prick and taken into heparinized capillary tubes. Subsequent processing of the plasma and immunofluorescent antibody tests were carried out according to the methods of Voller & O'Neill (4).

Results

One person could comfortably carry out tests on 2 plates (about 200 samples) per working day with the methods described. Qualitative visual readings of the results were easy to make and very rapid: 2 plates could be scanned within 5 min.

The absorbance readings (E400) on replicate samples were reproducible, indicating that the wells were uniformly coated with antigen.

The values for the two Colombian populations studied are shown in Fig. 1. They are much higher in most of the population of the malarious area, San Juan de Arama, than in the area Girardot, in which effective antimalarial measures have been taken.

Discussion

The microplate method described here is suitable for use in the field, where the results can be assessed visually, or in better-equipped laboratories, where spectrophotometric readings can be used for added sensitivity and discrimination. In many circumstances, the visual assessment of positive or negative results is all that may be required.

In the form described here, the test appears to be eminently suitable for large-scale seroepidemiological programmes. The plates are easy to handle and are relatively inexpensive. Only small quantities of reagents are required. It is possible to carry out the tests on extremely small samples of serum or plasma at a single dilution, so that assays for several different diseases could be carried out with a capillary tube of plasma and different plates sensitized with the appropriate antigens.

Possibly the main problem will be standardization, if comparable results are to be obtained at different times and by different laboratories. This can best be achieved by using reference preparations of positive and negative sera together with reference enzyme-labelled antiglobulin conjugates. In this way, different batches of antigen—which is the most variable factor—can be standardized.

The small seroepidemiological study of malaria is included to emphasize the practical aspects of this test. The assay values clearly indicate the differences in the malaria situation in Girardot and in San Juan de Arama.

As only small quantities of antigen were needed to sensitize the wells in the plates, we were able to use *P. falciparum* antigen, which is preferable to, but less readily available than, the simian malaria parasite *P. knowlesi* antigen used in earlier studies (2).

The fact that there is a positive correlation between the enzyme assay and indirect immunofluorescence augurs well for the new method, as the immunofluorescent antibody test—although it is

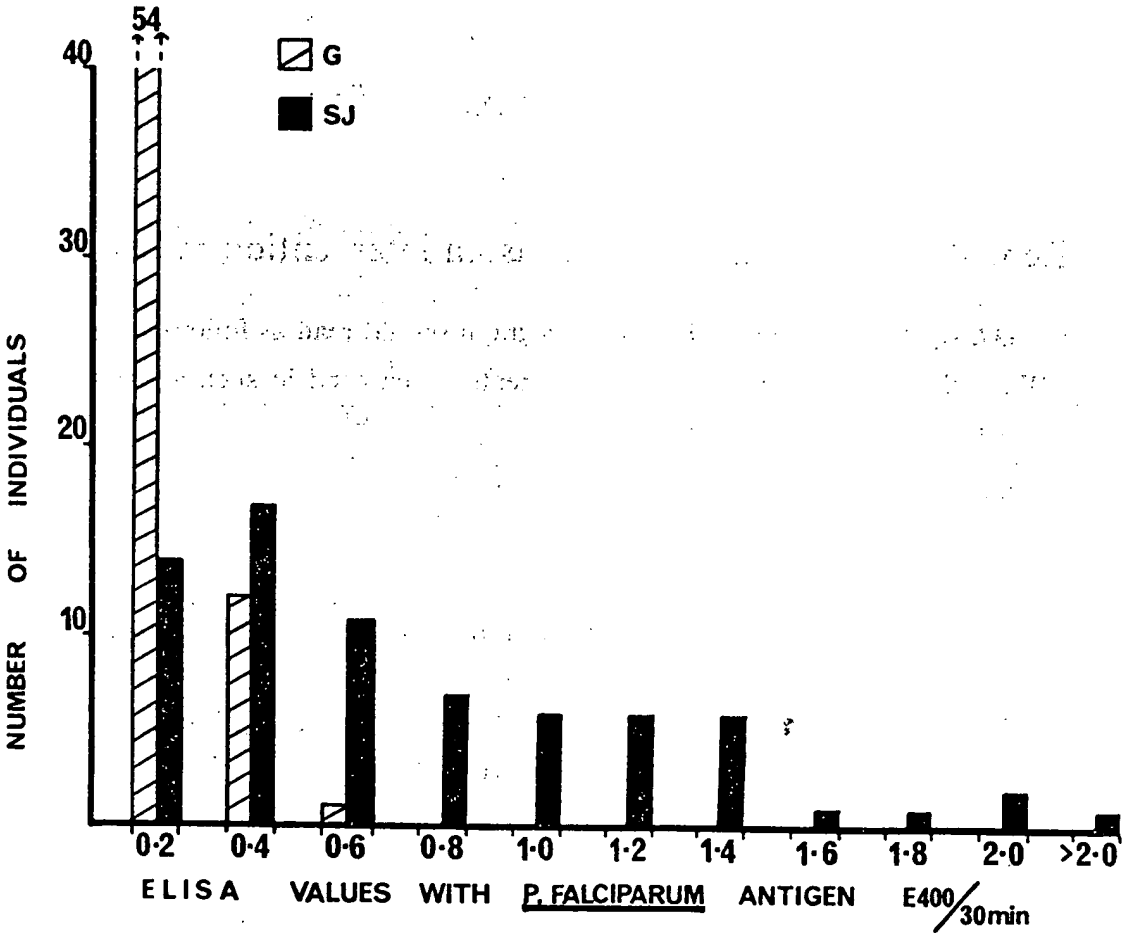

Fig. 1. ELISA values with *P. falciparum* antigen in two Colombian populations (G = Girardot—an area where an effective malaria eradication programme has been implemented; SJ = San Juan de Arama area, where malaria is endemic).

already accepted as a useful serological method—has the disadvantage of being very time-consuming.

The same method has been used, with promising results, in assays for antibody in Chagas' disease and in schistosomiasis.

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